



Growth and metabolism of non-*Saccharomyces* yeasts isolated from Washington state vineyards in media and high sugar grape musts

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ARTICLE INFO

Keywords:

Non-saccharomyces yeasts
Alcoholic fermentation
Amino acids
Wine

ABSTRACT

Utilization of carbohydrates and amino acids/ammonium by selected non-*Saccharomyces* yeasts and impacts on alcoholic fermentation was evaluated using media and high sugar grape musts (> 270 g/L). Consumption patterns of single cultures were ascertained in synthetic media and a Chardonnay grape must. While the non-*Saccharomyces* species maintained > 10⁶ cfu/mL after > 40 days, concentrations of residual sugars ranged from 103 g/L (*Wickerhamomyces anomalus*) to 155 g/L (*Candida californica*), amino acids and ammonium were generally depleted (> 85%), and excessive amounts of volatile acidity (> 0.8 g/L) were sometimes produced (e.g., *C. oleophila*). To minimize problems associated with nutrient depletion before alcoholic fermentation, non-*Saccharomyces* yeasts were inoculated six days ahead of *S. cerevisiae* into Syrah grape musts. Syrah ferments inoculated with *C. californica* or *Metschnikowia pulcherrima* contained lower concentrations of residual sugar and ethanol compared to those with only *S. cerevisiae*. Furthermore, the presence of non-*Saccharomyces* yeasts influenced concentrations of glycerol and volatile aroma compounds. These results suggested potential use of some non-*Saccharomyces* yeasts towards reducing alcohol concentrations without risking slower alcoholic fermentations.

1. Introduction

Yeasts found on wine grapes (*Vitis vinifera*) at the time of harvest are referred to as “native”, “wild”, or, more commonly, “non-*Saccharomyces*” (Fugelsang and Edwards, 2007). As could be expected, non-*Saccharomyces* yeasts comprise a heterogeneous group representing several genera such as *Aureobasidium*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Issatchenkia*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Wickerhamomyces*, and others (Bourret et al., 2013; Fleet, 1993; Jolly et al., 2003b; Li et al., 2010). Healthy, undamaged grape berries have reported viable populations which range from 10² to 10⁶ cfu/mL (Jolly et al., 2003b; Li et al., 2010).

The impacts of these yeasts on overall wine quality have long been the subject of debate. In fact, non-*Saccharomyces* yeasts can contribute either positively or negatively to the sensory profile of wines depending on a number of factors (Fleet, 2003; Jolly et al., 2003a; Viana et al., 2008). For instance, some species synthesize various esters responsible for desirable ‘fruity’ and ‘floral’ aromas (Izquierdo Cañas et al., 2014; Clemente-Jimenez et al., 2004; Viana et al., 2008; Zohre and Erten, 2002). Furthermore, some of these yeasts produce extracellular hydrolytic enzymes such as glucosidases, proteases, and/or pectinases

which can affect wine quality through enhancement of grape varietal aromas, mouthfeel, and/or other properties (Comitini et al., 2011; Cordero-Bueso et al., 2013). In contrast, other species produce undesirable surface films, turbidity, or excessive concentrations of ethyl acetate, acetic acid (volatile acidity or VA), and/or acetaldehyde that lessen or devastate quality (Andorrà et al., 2010; Ciani and Maccarelli, 1998; Rojas et al., 2003; Zohre and Erten, 2002).

Besides potentially altering sensory quality, growth of non-*Saccharomyces* yeasts can affect alcoholic fermentation conducted by *Saccharomyces cerevisiae*. On the one hand, non-*Saccharomyces* yeasts may consume key nutrients present in grape musts important for *S. cerevisiae* (Bataillon et al., 1996; Medina et al., 2012). As an example, Bataillon et al. (1996) noted that these yeasts can quickly deplete a grape must of thiamin, thereby causing stuck or sluggish fermentations. More recently, Medina et al. (2012) reported that *Hanseniaspora viniae* and *Metschnikowia pulcherrima* yeasts consumed enough nutrients to slow fermentations. Moreover, non-*Saccharomyces* yeasts may preferentially consume specific nitrogen sources, potentially impacting subsequent growth and fermentation by *S. cerevisiae* (Gobert et al., 2017; Kemsawasd et al., 2015; Rollero et al., 2018a, 2018b). On the other hand, co-inoculation of specific non-*Saccharomyces* yeasts may

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<https://doi.org/10.1016/j.fm.2018.09.004>

Received 18 May 2018; Received in revised form 28 August 2018; Accepted 3 September 2018

Available online 04 September 2018

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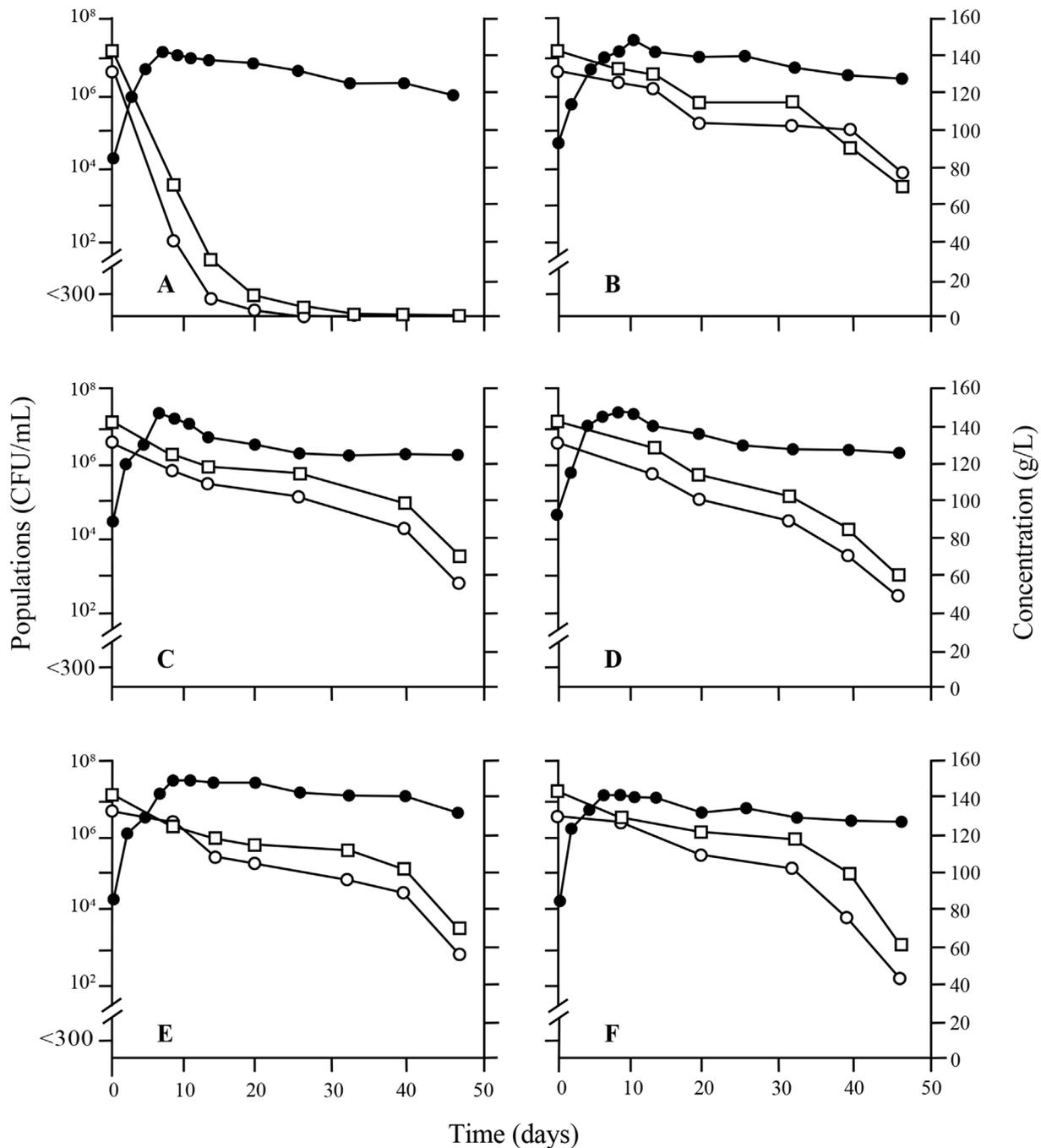


Fig. 1. Culturable populations (\bullet) and concentrations of glucose (\circ) and fructose (\square) in a Chardonnay must inoculated with *S. cerevisiae* (A), *C. californica* (B), *C. oleophila* (C), *Mt. pulcherrima* (D), *My. caribbica* (E), or *W. anomalus* (F).

reduce the risks of problem fermentations. As some species of non-*Saccharomyces* yeasts are fructophilic (Ciani and Fatichenti, 1999), co-inoculation with glucophilic *S. cerevisiae* (Berthels et al., 2004) may result in lower residual concentrations of fructose, reducing the risk of stuck alcoholic fermentations (Schutz and Gafner, 1995). Furthermore, Gonzalez et al. (2013) suggested that successive inoculation of yeasts can reduce the final alcohol contents of wines. Here, early inoculation of non-*Saccharomyces* yeasts transforms sugar to produce biomass and by-products besides ethanol before addition of *S. cerevisiae* (Quirós et al., 2014). This could prove particularly useful to winemakers souring grapes from hot, sunny regions where high sugar musts lead to wines exceeding 15% v/v alcohol (Mira de Orduña, 2010).

In a survey of grapes obtained from two vineyards located in Washington, 53 species of yeasts were isolated including *Candida*

californica, *C. oleophila*, *Mt. pulcherrima*, *Meyerozyma caribbica*, and *Wickerhamomyces anomalus* (Bourret et al., 2013). Frequently isolated from grapes, strains of *Mt. pulcherrima* have been identified that produce wines with reduced ethanol contents (Canonico et al., 2016; Contreras et al., 2014, 2015a; 2015b; Varela et al., 2016) and increased concentrations of fruit-associated esters (Clemente-Jimenez et al., 2004; Jolly et al., 2003a; Rodríguez et al., 2010; Zohre and Erten, 2002). Similarly, *W. anomalus* (synonym *Pichia anomala*) has also been isolated during fermentation (Fredlund et al., 2002) and enhances 'fruity' and 'floral' sensory properties (Izquierdo Cañas et al., 2014; Varela, 2016). Although few reports describe *C. californica* or *C. oleophila* on grapes, these species can metabolize glucose to varying degrees (Lachance et al., 2011). Another species, *My. caribbica* (synonym *Pichia caribbica*) was found to produce desirable volatile aromas in fermented pineapple

Table 1
Concentrations of amino acids and ammonium before and after 56 days of yeast growth in SGJM.

Nitrogen Compound	Pre-inoculation (mg N/L)	Post-inoculation (mg N/L)				
		<i>S. cerevisiae</i>	<i>C. californica</i>	<i>Mt. pulcherrima</i>	<i>My. caribbica</i>	<i>W. anomalus</i>
Ala	19.7	0.64 ^a	12.5 ^b	nd	nd	nd
Ammonium	58.2	nd	nd	nd	nd	nd
Arg	220	nd	nd	nd	nd	nd
Asp	6.31	2.65	nd	nd	nd	nd
Glu	11.9	2.46 ^a	4.27 ^b	nd	nd	nd
Gly	4.67	nd	1.56	nd	nd	nd
His	16.3	2.60 ^a	9.61 ^c	5.04 ^b	3.95 ^b	3.86 ^b
Ile	2.67	1.92 ^b	1.92 ^b	1.96 ^b	2.00 ^b	0.27 ^a
Leu	2.67	0.83 ^a	1.53 ^b	nd	nd	nd
Lys	4.79	5.04 ^a	5.02 ^a	4.76 ^a	5.16 ^a	4.84 ^a
Met	2.35	2.64 ^b	2.94 ^b	1.89 ^a	3.00 ^b	2.77 ^b
Phe	5.09	nd	3.57 ^b	nd	0.13 ^a	nd
Ser	16.6	nd	1.31	nd	nd	nd
Thr	14.7	3.04 ^a	3.31 ^a	nd	1.87 ^a	nd
Trp	8.23	nd	8.23	nd	nd	nd
Tyr	1.93	nd	nd	nd	nd	nd
Val	15.0	nd	nd	nd	nd	nd
Total	412	16.0 ^a	54.5 ^b	13.4 ^a	15.7 ^a	11.0 ^a

^{a-c} Post-inoculation mean values within a row with different superscripts are significantly different ($p \leq 0.05$).
nd: not detected.

beverages (Ribeiro et al., 2015).

While present on regionally-grown grapes, little is known regarding the impacts (if any) of the non-*Saccharomyces* isolates described by Bourret et al. (2013) on alcoholic fermentation or resultant wine quality. Furthermore, while nitrogen utilization by *Mt. pulcherrima* and *W. anomalus* have been reported (Gobert et al., 2017; Kemsawasd et al., 2015; Rollero et al., 2018b), consumption by *C. californica*, *C. oleophila*, and *My. caribbica* have not been previously characterized. Thus, the objectives of this study were to determine compositional changes to grape musts by several yeast isolates including consumption of sugars and amino acids as well as the production of various metabolites (VA, alcohol, and aroma/flavor compounds).

2. Materials and methods

2.1. Yeast strains

C. californica P01C003, *C. oleophila* P40C006, *Mt. pulcherrima* P01A016, *My. caribbica* P46A001, and *W. anomalus* P01A017 were previously isolated from vineyards located at the Irrigated Agriculture Research and Extension Center (Prosser, WA) as described by Bourret et al. (2013). *S. cerevisiae* strains ECA5 and D254 were obtained as active dry cultures from Lallemand Inc. (Montréal, Quebec, Canada). Strain ECA5 was selected given its low nitrogen requirements, while D254 is commonly used in commercial red wine fermentations (Cadière et al., 2011; G. Specht, personal communication). All yeasts were maintained on yeast peptone dextrose (YPD) agar slants.

To prepare starter cultures, single colonies grown on YPD agar were transferred to yeast/mold (YM) broth (Becton, Dickinson, and Company, Sparks, MD). Upon reaching late exponential growth, cells were harvested by centrifugation at $2000 \times g$ for 20 min and washed twice in 0.2 M Na_2HPO_4 (pH 7.0) buffer prior to inoculation.

2.2. Chardonnay grape must

Chardonnay grape must (pH 3.34, 133 g/L glucose, 141 g/L fructose, 137 mg N/L yeast assimilable nitrogen) was obtained from a regional winery. After removal of SO_2 by H_2O_2 , the must was sterile-filtered through 0.2 μm Nylon® membranes housed in an autoclaved filter housing (Pall, Port Washington, NY). Diammonium phosphate (50 mg/L) and powdered cellulose (1 g/L, Sigmacell® Type 20, Sigma-Aldrich,

St. Louis, MO) were aseptically added before transfer into sterile 100 mL milk dilution bottles. The cellulose was prepared as a suspension and sterilized at 121 °C for 15 min. Chardonnay musts were inoculated, in triplicate, with starter cultures of *C. californica*, *C. oleophila*, *My. caribbica*, *Mt. pulcherrima*, *S. cerevisiae* ECA5, or *W. anomalus* to achieve initial populations between 10^4 and 10^5 cfu/mL and incubated statically at 18 °C.

2.3. Synthetic grape juice medium

A synthetic grape juice medium (SGJM) was prepared without added sulfites as described by Wang et al. (2003). The medium was modified by adjusting the concentrations of glucose and fructose to 120 g/L each and of specific amino acid concentrations to 685 mg/L (Arg, Pro), 125 mg/L (Ala, Glu, Ser, Thr, Val), 60 mg/L (Asp, His, Phe, Try), or 25 mg/L (Gly, Ile, Leu, Lys, Met, Tyr) and 275 mg/L $(\text{NH}_3)_2\text{PO}_4$. The medium was sterile-filtered through 0.2 μm Nylon® membranes into previously sterilized 3L Celstir fermenters (Wheaton Science Products, Millville, NJ) equipped with aseptic syringe-type extraction ports (New Brunswick Scientific Co., Edison, NJ) that avoids air incorporation during sampling. Media was inoculated with starter cultures of *C. californica*, *Mt. pulcherrima*, *My. caribbica*, *S. cerevisiae* ECA5 and *W. anomalus* and incubated as described previously for the Chardonnay fermentations.

2.4. Syrah grape must

Syrah grapes were obtained from the Irrigated Agriculture Research and Extension Center (Prosser, WA). Potassium metabisulphite (10 mg/L SO_2) was added immediately after crushing/destemming and before storage of the must at -20 °C in 5-gallon containers. Upon thawing, the must (pH 3.51, 5.56 g/L titratable acidity, 152 g/L glucose, 149 g/L fructose, 253 mg N/L yeast assimilable nitrogen) was distributed into 1L sterile bottles after addition of Fermaid® K (0.33 g/kg) and potassium metabisulphite (30 mg/L SO_2). Bottles were either not inoculated or inoculated with *C. californica*, *Mt. pulcherrima*, *My. caribbica*, *S. cerevisiae* D254, *W. anomalus* at 10^5 cfu/mL in triplicate. Fermentation “water bubble” locks were added to musts inoculated with *S. cerevisiae* while uninoculated or those with non-*Saccharomyces* yeasts were sealed with gas-porous stoppers (Whatman® Bugstopper, Maidstone, U.K.). After six days, all musts were inoculated with *S. cerevisiae* D254 and sealed with

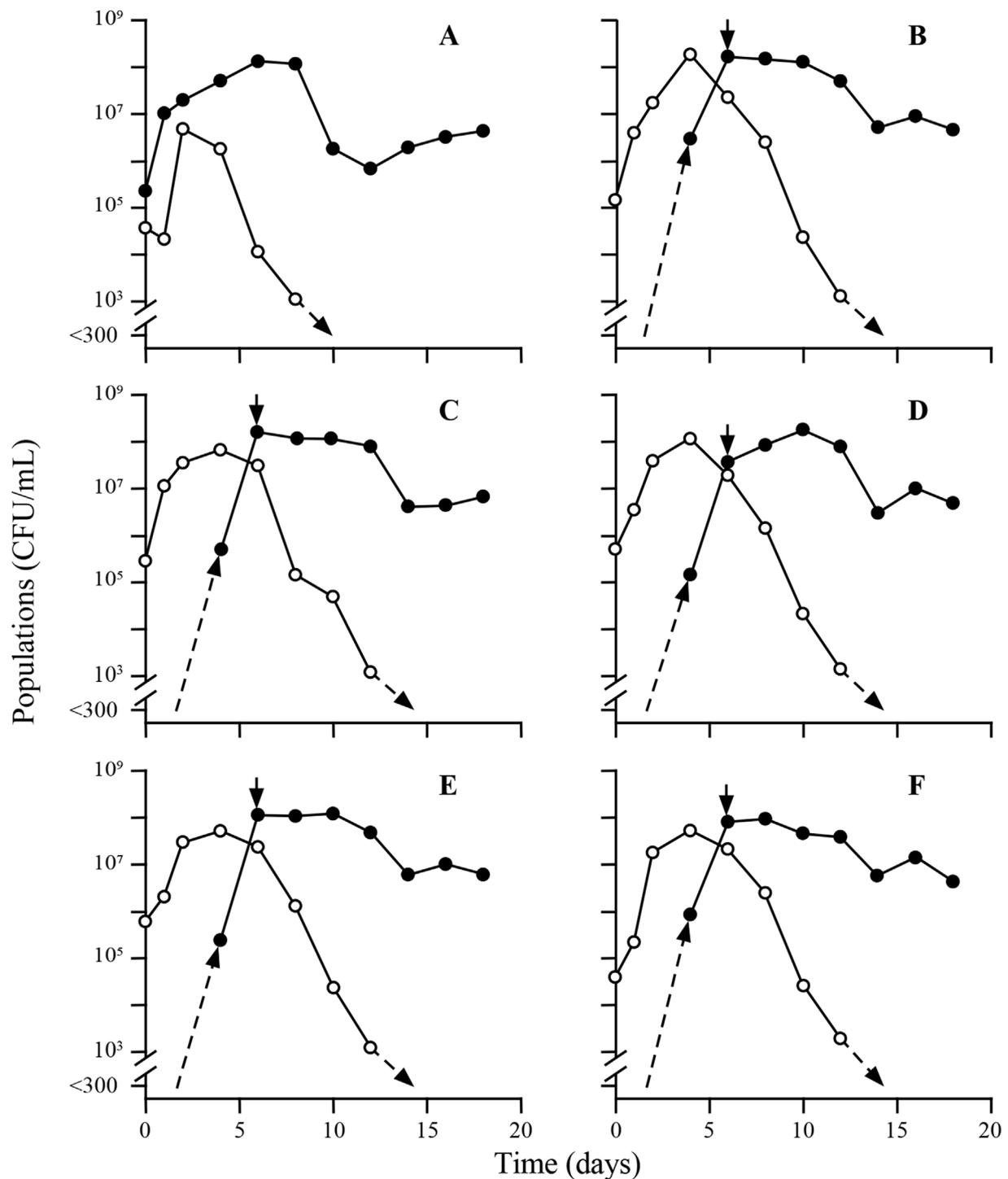


Fig. 2. Culturable populations of *Saccharomyces cerevisiae* (●) and non-*Saccharomyces* (○) yeast in a Syrah must inoculated either solely with *S. cerevisiae* D254 (A), sequentially with *C. californica* (B), *Mt. pulcherrima* (C), *My. caribbica* (D), or *W. anomalus* (E) followed by *S. cerevisiae* D254, or left initially uninoculated (F) before *S. cerevisiae* addition. The time of inoculation of D254 is indicated with an arrow.

fermentation locks with additional Fermaid[®] K (0.66 g/L) added two days later. Fermentations were conducted at 23 °C and stirred for 30 s each day. Once soluble solids reached 0° to 3°Brix, free-run wine was collected into sterilized milk dilution bottles to complete fermentation. Wines were considered “dry” when reducing sugar concentrations reached < 2 g/L as determined by the Clinitest[®] method (Ough and Amerine, 1988).

2.5. Analytical methods

Culturability was evaluated by spiral plating (Autoplate 4000, Spiral Biotech, Bethesda, MD) using either Wallenstein Laboratory agar (WL, Becton, Dickinson, and Company, Franklin Lakes, NJ) for total yeasts or lysine agar (Oxoid, Hampshire, England) for non-*Saccharomyces* yeasts after incubation for 3–5 days at 28 °C. Populations of *S. cerevisiae* in Syrah musts were estimated based on the difference between plate counts on WL and lysine agar media.

Amino acids, glycerol, and ethanol were quantified with an Agilent

Table 2

Chemical composition of Syrah wines produced by *S. cerevisiae* alone or by sequential inoculation with non-*Saccharomyces* followed by *S. cerevisiae* D254 six days later.

Inoculated Yeast	Glucose + Fructose (g/L)	Ethanol (% v/v)	Glycerol (g/L)	Titrateable Acidity (g/L)	Volatile Acidity (g/L)
<i>S. cerevisiae</i>	1.46 ± 0.506 ^b	16.4 ± 0.1 ^b	11.2 ± 0.1 ^a	6.84 ± 0.04 ^a	0.238 ± 0.005 ^a
<i>C. californica</i>	0.686 ± 0.260 ^{ab}	15.6 ± 0.3 ^a	12.0 ± 0.2 ^{ab}	7.31 ± 0.04 ^{bc}	0.286 ± 0.005 ^{bc}
<i>Mt. pulcherrima</i>	0.461 ± 0.058 ^a	15.5 ± 0.1 ^a	12.7 ± 0.1 ^b	7.61 ± 0.04 ^c	0.243 ± 0.008 ^{ab}
<i>My. caribbica</i>	0.554 ± 0.090 ^a	15.9 ± 0.2 ^{ab}	13.0 ± 0.8 ^b	7.19 ± 0.12 ^b	0.322 ± 0.009 ^c
<i>W. anomalus</i>	0.612 ± 0.077 ^a	16.4 ± 0.1 ^b	12.4 ± 0.1 ^{ab}	6.59 ± 0.12 ^a	0.442 ± 0.028 ^d
<i>S. cerevisiae</i> *	0.826 ± 0.159 ^{ab}	16.2 ± 0.1 ^b	12.6 ± 0.1 ^b	7.21 ± 0.18 ^b	0.315 ± 0.010 ^c

* Inoculated six days after crush.

^{a-d} Mean values within columns with different superscripts are significantly different ($p \leq 0.05$).

Table 3

Concentration of volatile compounds in Syrah wines inoculated initially with non-*Saccharomyces* or *S. cerevisiae* yeasts.

Compound (mg/L)	<i>S. cerevisiae</i>	<i>Mt. pulcherrima</i>	<i>My caribbica</i>	<i>C. californica</i>	<i>W. anomalus</i>
Higher Alcohols					
1-Propanol	4.00 ^a	8.33 ^b	13.1 ^{cd}	13.8 ^d	11.1 ^c
2-Methyl-1-propanol	32.5 ^a	140 ^c	106 ^b	152 ^c	95.3 ^b
2&3-Methyl-1-butanol	143 ^a	188 ^c	141 ^a	165 ^b	129 ^a
1-Hexanol	4.01 ^a	4.13 ^a	5.73 ^b	6.27 ^b	5.74 ^b
1-Octanol	2.11 ^a	3.81 ^a	2.56 ^a	4.08 ^a	0.76 ^a
2-Phenylethanol	30.4 ^{ab}	34.7 ^b	27.8 ^{ab}	26.0 ^{ab}	24.0 ^a
Esters					
Ethyl Acetate	69.3 ^a	168 ^b	297 ^c	344 ^{cd}	399 ^d
2&3-Methylbutyl Acetate	4.61 ^b	3.78 ^a	3.23 ^a	3.17 ^a	3.33 ^a
Hexyl Acetate	0.024 ^c	0.007 ^a	0.012 ^b	0.007 ^{ab}	0.011 ^b
Diethyl Succinate	0.208 ^a	0.309 ^b	0.193 ^a	0.317 ^b	0.205 ^a
2-Phenylethyl Acetate	0.150 ^a	0.165 ^a	0.157 ^{ab}	0.197 ^b	0.143 ^a
Ethyl Butanoate	0.428 ^b	0.409 ^b	0.316 ^a	0.273 ^a	0.306 ^a
Ethyl Hexanoate	0.202 ^{bc}	0.231 ^c	0.181 ^b	0.140 ^a	0.139 ^a
Ethyl Octanoate	0.518 ^c	0.422 ^{abc}	0.440 ^{bc}	0.343 ^a	0.381 ^{bc}
Acids					
Hexanoic Acid	3.56 ^a	6.72 ^c	5.04 ^{abc}	6.26 ^{bc}	4.11 ^{ab}
Octanoic Acid	2.98 ^{ab}	3.37 ^b	3.16 ^{ab}	3.24 ^b	2.71 ^a

^{a-d} Mean values within rows with different superscripts are significantly different ($p \leq 0.05$).

1100 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with UV-VIS diode array and refractive index detectors. Samples were filtered through 0.22 µm polyethersulfone membranes (MilliporeSigma) into crimp-top vials. Amino acids were quantified using an Agilent ZORBAX Eclipse Plus-C18 column (4.6 × 150 mm, 3.5 µm particle size) after pre-column *o*-phthalaldehyde derivatization according to Henderson and Brooks (2010). Yeast assimilable nitrogen was calculated as the sum of primary amino nitrogen, determined according to Dukes and Butzke (1998), and ammonium measured with an ammonia ion-selective electrode (Denver Instruments, Orville, NY, USA). Glucose and fructose concentrations for Chardonnay and Syrah fermentations were measured enzymatically (Yellowline Kit, r-Biopharm, Darmstadt, Germany) while volatile acidities were determined by Cash still (Ough and Amerine, 1988).

Volatile aromas were extracted by headspace solid-phase micro-extraction (HS-SPME) using a 65 µm polydimethylsiloxane-divinylbenzene fiber (Supelco, Bellefonte, PA) following the procedures of Clary et al. (2006). Compounds were analyzed with an Agilent HP 6890 gas chromatograph fitted with a 0.32 mm × 60.0 m, 1.0 µm thickness DB-1MS column (Phenomenex, Torrance, CA) coupled to a HP 5973 Mass Selective Detector. Fibers were desorbed for 3 min at 250 °C in the injection inlet operating in splitless mode. The carrier gas was helium flowing at 0.7 mL/min. The oven temperature was programmed to hold at 33 °C for 5 min, increase at 2.0 °C/min to 50 °C and then 5.0 °C/min to 225 °C and held for 5 min. The mass spectrometer was operating in electron impact mode at 70 eV (150 °C ion source). Compounds were identified using the Wiley/NIST library while quantification was accomplished using 4-methyl-2-pentanol and 2-octanol as internal

standards.

2.6. Statistical methods

Statistical analyses were performed by ANOVA using XLSTAT while mean separations were accomplished by Tukey's HSD (Addinsoft, New York, NY).

3. Results and discussion

3.1. Singular inoculation of non-*Saccharomyces* yeasts compared to *S. cerevisiae*

C. californica, *C. oleophila*, *Mt. pulcherrima*, *My. caribbica*, *S. cerevisiae* ECA5, and *W. anomalus* P01A017 were individually inoculated into a Chardonnay grape must to evaluate growth and sugar utilization under vinification conditions (Fig. 1). After approximately six days of growth, all yeasts attained populations $\geq 10^7$ cfu/mL and maintained $\geq 10^6$ cfu/mL for > 40 days. In agreement, Heard and Fleet (1988) and Cordero-Bueso et al. (2013) reported that *Mt. pulcherrima* and *W. anomalus* grew well in grape juice ($\geq 10^6$ cfu/mL) and remained viable for several weeks.

Even though maximum populations were similar, *S. cerevisiae* and non-*Saccharomyces* yeasts differed in sugar utilization. Although *S. cerevisiae* consumed most of the available glucose and fructose by day 30, those fermentations inoculated with non-*Saccharomyces* yeasts contained residual concentrations of 100–155 g/L glucose/fructose (Fig. 1; Supplementary Table S1). Those musts inoculated with *C.*

californica contained the highest concentrations of residual sugars (155 g/L) while those with *Mt. pulcherrima* (110 g/L) or *W. anomalus* (103 g/L) contained less. Lower sugar utilization by *Mt. pulcherrima*, *My. caribbica*, and *W. anomalus* compared to *S. cerevisiae* in grape musts was also observed by Cordero-Bueso et al. (2013), Gobert et al. (2017), and Jolly et al. (2003a). In contrast to some non-*Saccharomyces* species (Ciani and Faticenti, 1999; Magyar and Tóth, 2011), all yeasts preferred glucose compared to fructose. Growth and sugar utilization by *C. californica*, *C. oleophila*, or *My. caribbica* in a grape must had not been previously reported.

In the Chardonnay must, some of the non-*Saccharomyces* yeasts produced excessive concentrations of volatile acidity in contrast to *S. cerevisiae* (Supplementary Table S1). For instance, the highest concentration was found in musts inoculated with *C. oleophila* (0.825 g/L), an amount above a rejection sensory threshold established by Bandion and Valenta (1977). *W. anomalus* also yielded higher amounts (0.755 g/L) in compared to *S. cerevisiae* which only produced 0.38 g/L. These findings were in agreement with Rojas et al. (2003) who studied fermentation of red Bobal grapes by *W. anomalus* but in contrast to Cordero-Bueso et al. (2013) who used a white Malvar grape must. Based on high production of volatile acidity, further research involving *C. oleophila* was discontinued.

Nitrogen utilization by the non-*Saccharomyces* yeasts studied was determined through singular inoculation in SGJM after 56 days (Table 1). While populations reached between 10^6 and 10^7 cfu/mL (Supplementary Fig. S1), all yeasts depleted > 95% of available ammonium, arginine, tyrosine, and valine and lesser amounts of histidine, isoleucine, methionine, lysine, or other amino acid. The highest concentrations of residual amino acids were in media inoculated with *C. californica* (54.5 mg N/L). In fact, *Mt. pulcherrima*, *My. caribbica*, and *W. anomalus* consumed similar amounts of amino acids and ammonium compared to *S. cerevisiae*, a finding in agreement with Andorrà et al. (2010) who studied *Hanseniaspora uvarum* and *Starmarella bacillaris* (synonym *C. zemplinina*).

Given the high utilization of amino acids and ammonia when inoculated as pure cultures, the non-*Saccharomyces* yeasts studied could potentially deplete enough nutrients to hamper subsequent alcoholic fermentation by *S. cerevisiae*, in agreement with observations of others (Bataillon et al., 1996; Gobert et al., 2017; Medina et al., 2012; Rollero et al., 2018a, 2018b). However, experiments involving inoculation of single pure cultures were conducted over a long period of time (56 days). As such, it was possible that the non-*Saccharomyces* could metabolize some glucose and fructose without inhibiting alcoholic fermentation or producing excessive VA in grape musts if inoculated only a few days before *S. cerevisiae*.

3.2. Sequential inoculation of non-*Saccharomyces* followed by *S. cerevisiae*

As observed with other grape musts which were filter-sterilized prior to inoculation, non-*Saccharomyces* yeasts achieved high culturable populations in the unsterilized Syrah must (Fig. 2). Here, populations of *C. californica*, *Mt. pulcherrima*, *My. caribbica*, and *W. anomalus* exceeded $> 10^7$ cfu/mL. However, a low population of an unidentified strain of *S. cerevisiae* emerged on day 4 prior to subsequent inoculation of *S. cerevisiae* D254 on day 6. Non-*Saccharomyces* yeasts were not recoverable by day 12, an observation in agreement with others (Comitini et al., 2011; Contreras et al., 2014; Rodríguez et al., 2010).

Sugar consumption by *C. californica*, *Mt. pulcherrima*, *My. caribbica*, *S. cerevisiae*, and *W. anomalus* and concentrations of residual sugar, ethanol, and acetic acid in the resultant wines depended on the yeasts present (Table 2). Inoculation of non-*Saccharomyces* yeasts yielded dry wines by day 18 (< 2 g/L glucose and fructose) where those inoculated with *S. cerevisiae* alone contained 0.78–1.0 g/L more residual sugar than those with non-*Saccharomyces* yeasts. Regarding concentrations of ethanol after fermentation, wines produced with *C. californica* or *Mt. pulcherrima* contained less (0.8–0.9% v/v) than those fermented with

only *S. cerevisiae*. While Contreras et al. (2014; 2015a) and Varela et al. (2016) also reported decreases in ethanol production through inoculation of different strains of *Mt. pulcherrima*, this is the first report regarding the influence of *C. californica*. The presence of *My. caribbica* nor *W. anomalus* did not affect final alcohol contents, in contrast to Contreras et al. (2015b) studying other strains of the latter species.

Besides ethanol, growth of non-*Saccharomyces* yeast affected concentrations of glycerol as well as titratable and volatile acidities in the Syrah wines (Table 2). Wines produced with *Mt. pulcherrima* and *My. caribbica* contained 1.5 and 1.8 g/L more glycerol respectively than wines initially inoculated with *S. cerevisiae*. Inoculation of *C. californica*, *Mt. pulcherrima*, and *My. caribbica* increased titratable acidity compared to wines obtained from *S. cerevisiae* alone, partly due to differences in volatile acidities, similarly noted by Rodríguez et al. (2010) and Contreras et al. (2014). However, inoculation of these yeasts into the Syrah grape musts did not drastically increase resultant concentrations of volatile acidity (Table 2). In fact, concentrations were < 0.44 g/L for *W. anomalus*, in agreement with Rojas et al. (2003) and Cordero-Bueso et al. (2013). In mixed cultures, *S. cerevisiae* commonly dominates non-*Saccharomyces* yeasts within a short time period (Toro and Vazquez, 2002), thereby potentially lessening acetic acid production by these yeasts. However, some non-*Saccharomyces* yeasts were not influenced by *S. cerevisiae* as evidenced by Andorrà et al. (2010) who reported elevated concentrations inoculating *Hanseniaspora uvarum* or *Candida zemplinina* with *S. cerevisiae*.

Gonzalez et al. (2013) first suggested that sequential inoculation of Crabtree-negative non-*Saccharomyces* yeasts followed by *S. cerevisiae* could be used to reduce the final ethanol concentration of wines. Some of the non-*Saccharomyces* yeasts studied (e.g., *Mt. pulcherrima* or *W. anomalus*) are classified as Crabtree-negative where respiration is the preferred metabolic pathway regardless of glucose concentration (De Deken, 1966; Fredlund et al., 2002; Gonzalez et al., 2013; Schnierda et al., 2014). Such yeasts are often characterized by high affinity, energy-dependent glucose transport systems that result in slower accumulation of carbohydrates and less efficient conversion of sugar to ethanol (Van Urk et al., 1989). *S. cerevisiae* is Crabtree-positive and will exhibit fermentative metabolism regardless of the presence/absence of oxygen when the glucose concentration is > 9 g/L (Fugelsang and Edwards, 2007; Van Urk et al., 1989). In support, others have reported that the ethanol contents were reduced by 0.9–1.6% v/v when musts were inoculated with *Mt. pulcherrima* before adding *S. cerevisiae* (Canonico et al., 2016; Contreras et al., 2014; Varela et al., 2016). Varela et al. (2016) suggested that decreased alcohol content of fermentations by *Saccharomyces uvarum* were due to increased production of glycerol and succinic acid, although changes in glycerol and succinic acid content in Syrah fermentations (data not shown) were not sufficient to explain the observed ethanol reduction. To date, the metabolic preference of *C. californica* or *My. caribbica*, Crabtree-positive or negative, has not been studied.

Compared to wines inoculated with *S. cerevisiae* alone, the addition of non-*Saccharomyces* yeasts increased the amount of higher alcohols (i.e., fusel oils) in Syrah wines (Table 3). Concentrations of higher alcohols were greatest in wines with *Mt. pulcherrima* and *C. californica* present which produced more 2-methyl-1-propanol and 2- and 3-methyl-1-butanol than wines fermented solely with *S. cerevisiae*. Conversely, smaller increases in concentrations of 1-propanol (all species) and 1-hexanol (*C. californica*, *My. caribbica*, and *W. anomalus*) were noted in wines inoculated with non-*Saccharomyces* yeasts. Other authors have similarly noted increased concentrations of higher alcohols in wines made with *Mt. pulcherrima* (Contreras et al., 2014; Gobert et al., 2017) and *W. anomalus* (Izquierdo Cañas et al., 2014). Competition for specific nitrogen sources may explain the increased concentration of higher alcohols observed (Gobert et al., 2017; Rollero et al., 2018a) given their formation through transamination reactions involving amino acids (Fugelsang and Edwards, 2007). When present in moderate concentrations (< 400 mg/L), higher alcohols can contribute

beneficially to wine complexity (Rapp and Mandery, 1986).

Besides differences in higher alcohols, esters were more concentrated in Syrah wines inoculated initially with *S. cerevisiae* than those inoculated with non-*Saccharomyces* yeasts (Table 3). Primarily, *S. cerevisiae* wines contained increased concentrations of 2- and 3-methylbutyl acetate. While Comitini et al. (2011) and Varela et al. (2016) reported wines inoculated with *Mt. pulcherrima* to contain lower concentrations of esters, Contreras et al. (2014) and Izquierdo Cañas et al. (2014) noted that wines fermented with *Mt. pulcherrima* or *W. anomalus* had higher concentrations. Despite the elevated ester content however, *S. cerevisiae* wines may not be sensorily perceived as ‘fruitier’ compared to wines inoculated with *C. californica* and *Mt. pulcherrima* due to higher amounts of ethanol. In fact, increased concentrations of ethanol can decrease the perception of ‘fruity’ notes in wine (Goldner et al., 2009).

While non-*Saccharomyces* yeast can impart desirable sensory qualities, growth of these yeasts can also produce volatile compounds that negatively affect wine quality. Here, concentrations of such aroma compounds as ethyl acetate (‘nail polish remover’) and hexanoic acid (‘cheesy’) in wines with non-*Saccharomyces* yeasts were higher than in wines with *S. cerevisiae* alone (Table 3). Because ethyl acetate contributes unfavorably to the sensory profile of wines at concentrations above 150 mg/L, wines produced with non-*Saccharomyces* yeasts in this study will likely possess some negative sensory attributes (Fugelsang and Edwards, 2007; Rapp and Mandery, 1986). *W. anomalus* and *Mt. pulcherrima* have been reported to produce 200 mg/L ethyl acetate in Chardonnay and Bobal wines yet lower amounts in Shiraz (Contreras et al., 2014; Varela et al., 2016), suggesting an impact of specific winemaking practices on synthesis. In agreement, Rojas et al. (2001) observed greater ethyl acetate concentrations in aerated cultures of *P. anomala* (*W. anomalus*) while Comitini et al. (2011) noted increased ethyl acetate when the proportion of *S. cerevisiae* to non-*Saccharomyces* yeast inoculum was decreased.

4. Conclusions

This study examined the enological properties of five non-*Saccharomyces* yeast strains native to Washington vineyards during fermentations. For the first time, growth of *C. californica*, *C. oleophila*, and *My. caribbica* under winemaking conditions was described. In pure culture, non-*Saccharomyces* yeasts yielded high concentrations of residual sugar, consumed similar concentrations of ammonium and amino acids as *S. cerevisiae*, and, in some cases (i.e., *C. oleophila* and *W. anomalus*) produced significant amounts of acetic acid. When successively inoculated into Syrah must with *S. cerevisiae*, non-*Saccharomyces* yeasts increased concentrations of compounds beneficial to wine quality (titratable acidity, glycerol, and higher alcohols) without drastically increasing acetic acid content or fermentation time. Notably, *C. californica* and *Mt. pulcherrima* inoculation yielded wines with less alcohol, 0.8 and 0.9% v/v, respectively. In any case, the reduced alcohol wines made with *C. californica* or *Mt. pulcherrima* require sensory evaluation in order to fully evaluate the impact of these yeasts on wine quality in addition to optimizing processing conditions (i.e., temperature and oxygen availability).

Acknowledgements

The authors thank the Washington State Grape and Wine Research Program and the School of Food Science at Washington State University for financial and other support. Strains of *S. cerevisiae* were generously provided by G. Specht (Lallemand Inc., Montréal, Quebec, Canada). This research is part of project No. 0846 of the Agricultural Research Center, Washington State University, Pullman, WA, USA 99164–6376.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://>

doi.org/10.1016/j.fm.2018.09.004.

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